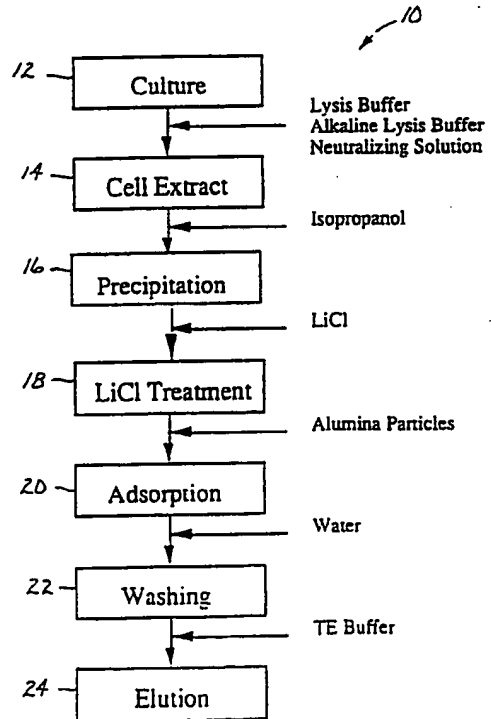


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US92/02262 <b>(22) International Filing Date:</b> 20 March 1992 (20.03.92) <b>(30) Priority data:</b> 685,125                      12 April 1991 (12.04.91)                      US <b>(71) Applicant:</b> MINNESOTA MINING AND MANUFACTURING COMPANY [US/US]; 3M Center, Post Office Box 33427, Saint Paul, MN 55133-3427 (US). <b>(72) Inventors:</b> KIM, Chan-Wha ; FUNKENBUSCH, Eric, F. ; MOH, Kyung, H. ; Post Office Box 33427, Saint Paul, MN 55133-3427 (US). <b>(74) Agents:</b> HORNICKEL, John, H. et al.; Minnesota Mining and Manufacturing Company, Post Office Box 33427, Saint Paul, MN 55133-3427 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> PURIFICATION OF NUCLEIC ACIDS USING METAL OXIDE SUPPORTS		
<div style="display: flex; justify-content: space-between;"> <div data-bbox="180 1136 812 2028"> <b>(57) Abstract</b>           Method and kits for the purification of nucleic acids incorporate metal oxide supports capable of preferentially sorbing nucleic acid from solution. The sorbed nucleic acid can thereafter be desorbed and eluted, or can be used in its sorbed state.       </div> <div data-bbox="812 1136 1513 2028"> <b>Purification of Plasmid DNA with Particle Suspension</b>    <pre> graph TD       12[Culture] --&gt; 14[Cell Extract]       14 --&gt; 16[Precipitation]       16 --&gt; 18[LiCl Treatment]       18 --&gt; 20[Adsorption]       20 --&gt; 22[Washing]       22 --&gt; 24[Elution]       10[10] --&gt; 12       13[13] --&gt; 14       15[15] --&gt; 16       17[17] --&gt; 18       19[19] --&gt; 20       21[21] --&gt; 22       23[23] --&gt; 24       </pre> <p>         12 Culture          14 Cell Extract          16 Precipitation          18 LiCl Treatment          20 Adsorption          22 Washing          24 Elution       </p> <p>         Lysis Buffer          Alkaline Lysis Buffer          Neutralizing Solution          Isopropanol          LiCl          Alumina Particles          Water          TE Buffer       </p> </div> </div>		

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PURIFICATION OF NUCLEIC ACIDS  
USING METAL OXIDE SUPPORTS

TECHNICAL FIELD

5           The present invention relates to methods and kits for the purification of nucleic acids, and more particularly, to insoluble supports useful in such methods, and to kits incorporating such supports.

10                   BACKGROUND OF THE INVENTION

          The purification of nucleic acids can involve, for instance, either the purification of one or more types of nucleic acids from other types of nucleic acids, or from other cellular materials.

15   Conventional methods for nucleic acid purification include the use of ultracentrifugation, phenol extraction, and the like. See, e.g., Basic Methods in Molecular Biology, L.G. Davis et al., eds., Elsevier, NY, 1986.

20           Of particular commercial and scientific importance, especially in the field of biotechnology, is the ability to purify plasmid DNA. Plasmids are small, generally circular, pieces of nucleic acid that can be modified by recombinant techniques to include  
25   gene sequences coding for proteins of interest. In the course of developing and using such "recombinant" plasmids it is highly desirable to be able to purify the plasmids from all other cellular constituents, including from cellular genomic (e.g., chromosomal)  
30   DNA.

          A number of nucleic acid purification methods have been described that involve the use of a solid support, e.g., for the immobilization of the desired nucleic acid, or for the removal of the unwanted  
35   cellular components. U.S. Patent No. 4,923,978, for instance, describes the use of a rehydrated silica gel capable of binding enzymes so as to leave nucleic acids unbound. U.S. Patent No. 4,935,342 describes a method

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for isolating or purifying nucleic acids that involves the use of particular anion exchange materials and control of the molarity of the various solutions used.

U.S. Patent No. 4,648,975 describes silicon-  
5 based chromatographic and reactive materials with surfaces modified to contain or to be coated with oxides, hydrous oxides, hydroxides, carbonates, or silicates of aluminum, iron, or other suitable metals such as zirconium or titanium. The modified support  
10 can be used as a chromatographic support for separating nucleosides, nucleotides, and nucleic acids.

European Patent Publication 0 391 608 describes metal oxide supports and compositions of metal oxides and nucleic acids that are useful, for  
15 example, in methods for the hybridization of nucleic acids.

Presently available supports suffer from various drawbacks however. Such drawbacks can include the need to use high salt concentrations for nucleic  
20 acid elution, long procedures to remove other cellular constituents such as protein or RNA, and the loss of nucleic acids during alcohol precipitation. What is clearly needed is a support useful for the purification of nucleic acids that avoids these and other drawbacks  
25 of presently available supports.

#### SUMMARY OF THE INVENTION

The present invention provides a method for  
30 the purification of nucleic acids comprising the steps of

- (1) releasing substantially all cellular nucleic acids from a biological sample into solution, and
- 35 (2) combining the solution with a metal oxide support under conditions in which nucleic acid preferentially sorbs to the support.

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Preferably the nucleic acid that sorbs to the support is or includes the desired nucleic acid. In the alternative, the nucleic acid that sorbs can include undesired types of nucleic acid(s), in such a manner  
5 that the desired nucleic acid thereby remains in solution in a more purified form, at least insofar as it has now been separated from the sorbed nucleic acid.

In another aspect, the present invention provides a kit for the purification of nucleic acids.  
10 The kit includes means for lysing cells in suspension in order to release substantially all nucleic acid into solution, together with a metal oxide support capable of sorbing released nucleic acid.

Supports of the present invention, if sorbed  
15 with a desired nucleic acid, are useful in a variety of ways, e.g., as chromatographic columns. Such supports provide a particular advantage in that the sorbed nucleic acids can thereafter be readily desorbed at will. Optionally therefore, the method also includes  
20 the steps of washing the sorbed support, then desorbing and eluting the nucleic acid.

The method is useful for any biological or other sample containing the desired nucleic acid, e.g., where the desired nucleic acid is present in  
25 combination with unwanted biomolecules such as other cellular components. The method can be used, for instance, for the purification of a desired nucleic acid from eucaryotic cells, or from procaryotic organisms such as animal and bacterial cells, yeast  
30 cells, and viruses.

The present invention provides an optimal combination of such properties as recovery, relative purity, and biological activity of the recovered nucleic acid, as well as versatility, cost, speed,  
35 simplicity, and ease of use.

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## BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 depicts a flow diagram for the purification of plasmid DNA from a cell suspension.

FIG. 2 depicts a flow diagram for the recovery of fragmented DNA from a gel.

FIG. 3 is a graph depicting sorption of calf thymus DNA on zirconia particles.

FIG. 4 is a graph depicting sorption kinetics of calf thymus DNA on zirconia particles in deionized water.

FIG. 5 is a graph depicting sorption kinetics of calf thymus DNA on alumina particles in deionized water.

FIG. 6 is a graph depicting selective sorption of calf thymus DNA on alumina particles from a mixture with bovine serum albumin ("BSA").

FIG. 7 is a graph depicting recovery yield of DNA from a mixture with BSA using zirconia particles.

FIG. 8 is a graph depicting recovery yield of RNA from a mixture with BSA using alumina particles.

## DETAILED DESCRIPTION

The present invention discloses that metal oxide supports, such as those described in European Patent Publication 0 391 608 can also be used to purify all, or a desired type, of the nucleic acids, e.g., from a cell suspension or other solution or from another support, such as a gel. The method is adaptable to the purification of any desired nucleic acid, including linear or circular plasmid DNA, chromosomal DNA, or RNA.

As used herein, the word "purify", and inflections thereof, refers to the separation of one physical or chemical type of nucleic acid from one or more other types within a sample such as a biological sample, such as DNA from RNA, or circular plasmid DNA from chromosomal DNA. The word also refers to the

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separation of nucleic acids from non-nucleic acid biomolecules such as the proteins, lipids, and other components making up the sample. The purification of nucleic acids according to this invention can therefore  
5 include both the purification of nucleic acids from complex solutions such as cell lysates and cell extracts, as well as the clarification of nucleic acid-containing solutions, such as those prepared in the course of in vitro syntheses of nucleic acids.

10 In the EXAMPLES below, purity is reflected in three alternative ways, taking into account either: (1) the recovery yield of nucleic acid, i.e., the amount (e.g., mass) of recovered nucleic acid divided by the total amount of the same nucleic acid present in the  
15 sample; (2) the relative purity, i.e., the amount of the nucleic acid recovered divided by the amount of other nucleic acids and/or biomolecules in the sample; and (3) the biological activity of the recovered nucleic acid, i.e., the ability of the nucleic acid to  
20 function in the desired biological manner. The term "nucleic acid(s)", as used herein, refers to deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA"), including various nucleic acid "types" such as circular or linear plasmid DNA and RNA, genomic DNA and  
25 RNA, and fragmented DNA and RNA (e.g., nucleic acid fragments that have been separated as one or more bands in a gel). The term therefore refers to any nucleic acid type or combination of nucleic acid types that are biochemically or physically separable from other types  
30 or combinations, e.g., by preferential precipitation, conventional sorption techniques, or gel separation.

The present invention will be more fully described below in reference to the flow charts 10 and 30 shown in FIGS. 1 and 2. In FIG. 1 there is outlined  
35 a presently preferred method for the purification of plasmid DNA from a cell suspension. In FIG. 2 there is outlined a presently preferred method for the

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purification of separated, fragmented DNA from a gel band.

As can be seen in the first steps of FIG. 1, the preferred method begins with the release from  
5 procaryotic cell culture 12 of substantially all nucleic acids from the cell into solution, e.g., by lysing or extracting the cells to create cell extract 14. Any suitable procaryotic, eucaryotic, or other suspension can be used, as long as it contains the  
10 desired nucleic acid.

The cells can be lysed or extracted in any manner, e.g., chemical, physical, and/or biological that is suitable to release into solution at least the desired type(s) of nucleic acid. In FIG. 1 the cells  
15 are portrayed as being completely lysed, thereby releasing not only the desired plasmid DNA, but substantially all other cellular components as well. Lysis in this particular case is preferably accomplished by the addition of an alkaline lysing  
20 solution sufficient to increase the pH of the sample to between about pH 12 and about pH 13. The alkaline lysing solution also preferably includes sodium dodecyl sulfate ("SDS"), in order to precipitate cellular RNA and protein.

25 The lysing solution is added in an amount, concentration, and under conditions suitable to provide the desired extent of lysis of the cells. Preferably, when using alkaline conditions, the lysing solution is then "neutralized", i.e., further lysis is terminated  
30 in order to allow chromosomal DNA, large molecular weight RNA, and protein to be precipitated. For instance, an alkaline lysing solution as described above can be neutralized by potassium acetate. See, e.g., H.C. Birnboim, Meth. Enzymol., 100:243-255  
35 (1983).

As a further step in the approach of FIG. 1, the unwanted RNA is substantially removed from solution by any suitable means within the skill of those in the



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art, e.g., by the use of an RNase enzyme capable of digesting the RNA, or, preferably, by lithium chloride (LiCl) precipitation (18) of the RNA. In a preferred embodiment the solution is subjected to an alcohol (e.g., isopropanol) precipitation (16) prior to LiCl treatment. The alcohol precipitates large RNA and plasmid DNA, leaving smaller RNA in solution. The pellet can then be resuspended in fresh buffer and the LiCl used to precipitate the large RNA alone, thereby leaving plasmid DNA in solution.

As a further step in the approach of FIG. 1, the desired nucleic acid, which in the present diagram would still be substantially intact and in solution, is sorbed (20) to a metal oxide support, which in turn is then removed from the solution, thereby leaving other impurities, such as proteins and salts, in solution. The word "sorb", and inflections thereof, is used interchangeably with the word "adsorb" herein, and refers to the attachment of nucleic acid to a metal oxide support by physical and/or chemical interactions when the nucleic acid and support are combined in solution according to the method of the present invention. The sorbed nucleic acid can thereafter be washed (22) and, if desired, desorbed and eluted (24) as described more fully below.

FIG. 2 depicts a flow diagram (30), discussed more fully in the EXAMPLES below, for the recovery of fragmented DNA from a gel.

Suitable metal oxides of the present invention exhibit an optimal combination of such properties as strength of sorption and sorption capacity, with respect to nucleic acids. The term "metal oxide" as used herein refers collectively to metal oxides and hydroxides as well as hydrous metal oxides and hydroxides as that term is defined and used in European Patent Publication 0 391 608. The word "hydrous" as used herein refers to metal oxide or hydroxide surfaces containing physically and/or

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chemically adsorbed water, and will be used in parentheses herein to indicate the optional presence of such water.

"Strength" of sorption, as used herein, refers to the ability of sorbed nucleic acid to remain sorbed to the metal oxide for purposes of the intended use of the resulting composition. "Sorption capacity" of the metal oxide, refers to the ability of the particular metal oxide support to sorb enough nucleic acid, per unit weight or surface area of the support, to purify the solution and/or for the subsequent intended use of the resulting composition. In turn, it relates to the amount of nucleic acid that a particular metal oxide can sorb per unit weight or surface area.

Examples of suitable metal oxides include, but are not limited to, the (hydrous) oxides and hydroxides of: calcium, cobalt, hafnium, iron, lanthanum, magnesium, manganese, nickel, titanium, yttrium, and zinc. When it is desired that supports of the present invention provide magnetic properties, preferred metal oxides include those of iron, cobalt, and nickel.

Examples of preferred metal oxides include, but are not limited to, the oxides and hydroxides of zirconium and aluminum, including in particular zirconia ( $\text{ZrO}_2$ ) and alumina ( $\text{Al}_2\text{O}_3$ ). Of the various phases of alumina, particularly preferred are compositions that are of predominantly the alpha alumina form.

Alumina particles can be prepared by methods known to those skilled in the art, for example by spray drying or atomizing. See, e.g., U.S. Patent No. 4,931,414. In order to obtain particles having desired and reproducible particle diameter distribution and size, it is preferred to prepare such particles by an emulsion technique such as that described further below.

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Preferably the accessible (i.e., to the desired nucleic acid(s)) surface area of a support is greater than about 1 m<sup>2</sup>/g metal oxide, especially about 3 m<sup>2</sup>/g, and particularly preferred are supports having 5 surface areas of greater than about 5 m<sup>2</sup>/g metal oxide. Surface area can be determined in any suitable fashion. For purposes of the present invention, surface area is determined by nitrogen absorption, as described in Absorption Surface Area and Porosity, S.J. Gregg and 10 K.S.W. Sing, Academic Press, London and New York (1967).

Metal oxide can be formed into any suitable support. For instance, metal oxide particles can be used in suspension or packed in a column when the 15 nucleic acid is to be sorbed from aqueous suspension, or in the form of a colloidal particle suspension, coating, or composite structure (e.g., membrane) incorporating particles. Those skilled in the art will recognize that the particle size will vary depending on 20 the form of the support, e.g., when packed in a column, metal oxide particles having an average diameter of about 5 μm to about 500 μm will generally be used, and preferred will be particles of about 10 μm to about 200 μm. For use in suspension, metal oxide particles having 25 an average diameter of about one-tenth μm to about 50 μm will generally be used, and preferred are particles of about one μm to about 10 μm. For use in a composite structure, such as an extruded, woven, or non-woven web, metal oxide particles having an average diameter 30 of about 1 μm to about 500 μm will generally be useful, with particles about 10 μm to about 100 μm being preferred.

The purified nucleic acid can be used for any suitable purpose, e.g., if still sorbed to a support 35 the nucleic acid can be used in that form, for instance, as a chromatographic or otherwise reactive column.

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Optionally the support with sorbed nucleic acid can be washed to remove nonspecifically bound proteins and salts, e.g., with water or sodium or potassium chloride solutions. The plasmid DNA can be  
5 desorbed, eluted and recovered from the support with a suitable elution buffer, e.g., by the use of Tris(hydroxymethyl)aminomethane hydrochloride ("Tris") - ethylenediamine tetraacetic acid ("EDTA") buffer (e.g., 5 to 30 mM Tris and 0 to 5 mM EDTA and about pH  
10 7 to about pH 9) or phosphate buffer (e.g., 10 to 50 mM and about pH 6 to about pH 8).

The present invention also provides a kit for the purification of nucleic acids. The kit incorporates both means for lysing a cell suspension as well as a  
15 metal oxide support capable of sorbing the released nucleic acid. Lysing means can include any suitable physical, chemical, and/or biological (e.g., enzymatic) method. Preferably lysing means include the combination of a lysis buffer to suspend the cells, together with  
20 an alkaline lysis reagent in order to actually disintegrate (i.e., rupture or lyse the cells), and a neutralizing solution in order to precipitate the desired nucleic acid (e.g., chromosomal DNA).

An example of a suitable lysis buffer is a  
25 combination of Tris (e.g., 10 mM to 50 mM): EDTA (e.g., 1 mM to 20 mM): glucose (e.g., 0 mM to 100 mM) in solution. A suitable alkaline lysis reagent is the combination of base, such as NaOH on the order of about 0.1 N to about 0.5 N, together with a surfactant such  
30 as sodium dodecyl sulfate ("SDS"), on the order of about one-tenth to about two percent by weight, based on the weight of the reagent. A suitable neutralizing solution is potassium acetate, e.g., on the order of 2 Molar to 4 Molar.

35 Objects and advantages of this invention are further illustrated by the following EXAMPLES, but the particular materials and amounts thereof recited in these EXAMPLES, as well as other conditions and

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details, should not be construed to unduly limit this invention. Unless otherwise indicated all percentages are by weight/volume.

#### EXAMPLES

5

##### EXAMPLE 1

##### Optimum Concentration of Metal Oxide Particles

A 4500 g sample of "Nyacol Zr 100/20" a colloidal  $\text{ZrO}_2$  sol manufactured by Nyacol Inc., Ashland, Massachusetts and containing 20% (by weight) of  $\text{ZrO}_2$  particles primarily of about 100 nm in size was concentrated on a rotary evaporator until its concentration was about 35%  $\text{ZrO}_2$  by weight. This sol was then spray dried on a spray dryer manufactured by Nyro Inc. About 900 g of dried spherical particles were obtained ranging in size from about 0.5  $\mu\text{m}$  to about 30  $\mu\text{m}$ . The dried zirconia particles were heated in a furnace to a temperature of 600°C over 6 hours and held at 600°C for 6 more hours. The furnace was then turned off and allowed to cool. The resulting fired spherical  $\text{ZrO}_2$  particles were then air classified and fractions from about 1  $\mu\text{m}$  to about 5  $\mu\text{m}$  or about 10  $\mu\text{m}$  to about 20  $\mu\text{m}$  in diameter were used in subsequent experiments.

To 1.5 ml of 0.005% (weight/volume in deionized water) calf thymus DNA in small centrifuge tubes (Eppendorf<sup>TM</sup>) were added increasing concentrations of the particles. The tubes were then mixed for 10 minutes. The samples were then centrifuged for 5 minutes at 14,000 rpm. The supernatant was sampled to measure the absorbance at 260 nm to determine the DNA still in solution. The entire method described was performed at ambient temperature. The results are shown in FIG. 3. Plot A represents the 1-5  $\mu\text{m}$  particles and plot B represents the 10-20  $\mu\text{m}$  particles. As is clear from the results, DNA sorbed to the zirconia particles in every tube in which the two were present. Nearly all DNA in the solution can be sorbed to such particles, depending on the amount, size, and accessible surface area of the particles.

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EXAMPLE 2Sorption Kinetics of DNA on Metal Oxide Particles

To multiple 1.5 ml 0.005% calf thymus DNA in Eppendorf<sup>TM</sup> centrifuge tubes, zirconia particles as described in EXAMPLE 1 or alumina particles (average particle size 2  $\mu$ m in diameter, prepared as described in EXAMPLE 4 below) were added to a concentration of either 200 g particles/g DNA or 400 g particles/g DNA, with mixing. The tubes were then incubated at room temperature with gentle shaking. At various time intervals sample tubes were centrifuged for 5 minutes at 14,000 rpm and the supernatant was sampled. The absorbance at 260 nm was measured to determine the DNA still in solution.

As is clear from the results shown in FIGS. 4 and 5, sorption of DNA on zirconia or alumina particles is substantially complete within 1 minute. In FIG. 4, plot C represents 200 g zirconia/g DNA and plot D represents 400 g zirconia/g DNA. In FIG. 5 plot E represents 4 g alumina/g DNA and plot F represents 20 g alumina/g DNA. Therefore, recovery of DNA by sorption on the metal oxide particles is sufficiently rapid to allow its use in routine bioseparation processes.

EXAMPLE 3Selective Sorption of DNA on Metal Oxide Particles

To 1.5 ml 0.005% calf thymus DNA and 0.05% bovine serum albumin ("BSA") in increasing concentrations of NaCl solutions, alumina particles such as those described in EXAMPLE 4 (400 g alumina/g DNA) were added. The mixtures were shaken for 10 minutes and centrifuged for 5 minutes at 14,000 rpm. The supernatant was sampled and the absorbance of the samples was measured at 260 and 280 nm to determine the DNA and protein remaining. The adsorption of DNA and BSA was determined from the concentration difference in the supernatant.

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FIG. 6 shows the effects of NaCl on the selective sorption of DNA on alumina particles. Plot G represents DNA and plot H represents BSA. When the concentration of NaCl is higher than about 200 mM, greater than 99% of DNA in the solution sorbed on the alumina particles, leaving greater than 90% of BSA in solution. Therefore, alumina particles selectively sorbed DNA from the mixture. Such selective absorption was also demonstrated using zirconia particles.

10

#### EXAMPLE 4

##### Comparison of Alumina/Aluminum Oxide Particles

Four different particles were compared as described below. The following particles were used:

15

(A) Unisphere<sup>TM</sup>-N (8  $\mu$ m normal phase alumina, Catalog # A-301-01000, Biotage, Inc., Charlottesville, VA)

(B) Alumina Woelm<sup>TM</sup> N (10-18  $\mu$ m neutral alumina particles) catalog # 402152, ICN Pharmaceuticals GmbH & Co., Germany

25

(C) Aluminum oxide (44  $\mu$ m particles, Catalog # 34,268-8, Aldrich Chemical Co., Milwaukee, WI)

(D) alumina particles having high alpha alumina were prepared by an emulsion technique as described below.

Particles (D) were prepared in the following manner:

- 1) a sol precursor "A" was prepared from 0.019 g of magnesium nitrate (reagent grade magnesium nitrate hexahydrate, MCB Manufacturing Chemicals, Inc., Cincinnati, OH) dissolved in 59.7 grams of colloidal alumina sol (Nalco<sup>TM</sup> #614 10% solids in aqueous solution, Nalco Chemical Co., Naperville, IL);
- 2) 10 g of emulsifier (sorbitan sesquioleate, "Arlacel<sup>TM</sup>" 83, Emulsion Engineering

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Inc., Sanford, FL) was stirred into 130 g of peanut oil and placed into a commercial blender mixing flask; then precursor A was slowly added to the emulsifier/oil solution as it was blended in order to emulsify it;

5                   3) the emulsified solution from the blender was poured into a microfluidizer apparatus (Microfluidics Corp., Newton, MA), which served to further break up the emulsion droplets; this solution was fed from the microfluidizer chamber into a  
10 container of hot (95°C), stirring oleyl alcohol which served to gel the sol precursor; this solution remained stirring until all of the emulsion solution had been added, and then was stirred for about 30 minutes longer until the solution cooled sufficiently to handle;

15                   4) the gel particles formed in the oleyl alcohol were collected by suction filtering (using aspirator pressure) the oleyl alcohol through Whatman No. 2 filter paper;

5) the collected gel particles were mixed  
20 with highly purified wood cellulose (Solka Floc<sup>TM</sup>, James River Corp., Berlin, NH) at a volume ratio of 1:1, this mixture was added to ethanol at a volume ratio of about 1:2 (particles to ethanol) and then filtered through Whatman #2 filter paper using  
25 aspirator pressure; the wood cellulose was added in this step to facilitate filtering and to prevent agglomeration during firing;

6) the gel particles were prefired (to burn off organic constituents) at 950°C, using the following  
30 heating schedule:

Room temperature to 950°C at 50°C/hour

950°C to hold for 1 hour

950°C to room temperature in air

7) the prefired particles were fired at  
35 1400°C, by ramping to 1400°C at 47°C/hour, held at 1400°C for 10 minutes, and cooled in the furnace to room temperature.



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The following properties were evaluated using the four types of particles:

TABLE 1

Properties	Particles			
	A	B	C	D
XRD	$\gamma\text{-Al}_2\text{O}_3$ $\theta\text{-Al}_2\text{O}_3$ $\beta\text{-Al}(\text{OH})_3$	$\eta\text{-Al}_2\text{O}_3$ $\beta\text{-Al}(\text{OH})_3$	$\alpha\text{-Al}_2\text{O}_3$ $\beta\text{-Al}(\text{OH})_3$ $\theta\text{-Al}_2\text{O}_3$	$\alpha\text{-Al}_2\text{O}_3$ $\beta\text{-Al}_2\text{O}_3$
Density (g/cc)	1.71	3.22	3.62	4.09
Surface area (m <sup>2</sup> /g)	49.0	152	0.41	2.96
Size (μm)	8~10	10~40	20~80	2~3
Shape	spherical	irregular	sharp edges	spherical

20

In TABLE 1 X-ray diffraction ("XRD") was performed using an automatic powder diffractometer (Philips "APD 3600", Philips Electronic Instruments, Inc., Mahwah, NJ). The identified metal oxide phases were the predominant species and are listed in decreasing order of peak intensity.

As can be seen, the particles (A) were predominantly gamma alumina with a minor amount of theta alumina and trace amounts of beta aluminum hydroxide. The particles (B) were predominantly eta alumina with minor amounts of beta aluminum hydroxide. The particles (C) were predominantly alpha alumina with minor amounts of beta aluminum hydroxide and trace amounts of theta alumina, and the particles (D) were predominantly alpha alumina with minor amounts of beta alumina, and were substantially devoid of aluminum hydroxide.

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Surface area was determined by conventional Brunauer-Emmett-Teller ("BET") nitrogen adsorption technique using a Quantasorb<sup>TM</sup> model "SW-6" surface area measuring instrument (Quantachrome Corp. Syosset, NY). Density was determined by helium pycnometry using a Quantachrome Stereo Pycnometer (Quantachrome Corp.). Size and shape were determined by visual examination using transmission light microscopy.

The four type of particles were compared in both particle suspension and minicolumns for the purification of plasmid DNA according to the method diagrammed in FIG. 1. The results are provided in TABLE 2 below. Unless otherwise indicated in these EXAMPLES, adsorption yield and recovery yield were determined using absorbance at 260 nm ( $A_{260}$ ) as follows:

$$\text{Adsorption Yield (\%)} = \frac{A_{260} \text{ initial} - A_{260} \text{ remaining}}{A_{260} \text{ initial}} \times 100$$

$$\text{Recovery yield (\%)} = \frac{A_{260} \text{ recovered}}{A_{260} \text{ initial}} \times 100$$

<u>TABLE 2</u>			
		<u>Adsorption</u>	<u>Recovery</u>
		<u>Yield</u>	<u>Yield</u>
30	<u>Alumina Particles</u>		
	<u>Particle Suspension</u>		
	A	90%	0%
	B	88%	0%
	C	74%	3%
35	D	91%	93%
	<u>Minicolumn</u>		
	A	91%	2%
	B	97%	2%
	C	62%	14%
40	D	92%	83%

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As can be seen, each of the particles exhibited significant adsorption of plasmid DNA. The somewhat lower adsorption of particles (C) may be due to their larger particle size (average about 44  $\mu$ m diameter).

In terms of the recovery of DNA after desorption and elution, the alumina particles (D) exhibited substantially total recovery under the particular conditions employed.

10

#### EXAMPLE 5

##### Desorption of DNA Sorbed on Metal Oxide Particles

Calf thymus DNA (0.005%) was sorbed on zirconia particles prepared as described in EXAMPLE 1 (400 g particles/g DNA) from a mixture with BSA (0.05%) at 200 mM NaCl in the manner described in EXAMPLE 3. After centrifugation the supernatant was discarded and the pellet was resuspended in 200 mM NaCl to wash out non-specifically bound BSA. The samples were centrifuged for 5 minutes at 14,000 rpm and the supernatant was discarded. The pellets were resuspended in increasing concentrations of potassium phosphate (pH 7.0) and shaken for 30 minutes. The mixtures were centrifuged for 5 minutes at 14,000 rpm. The supernatants were sampled and absorbance at 260 and 280 nm were measured to determine the concentrations of DNA and BSA. The recovery yield of DNA and BSA was determined as the concentration difference from the initial concentration as in

30 EXAMPLE 4.

The results are shown in FIG. 7. Plot I represents DNA and plot J represents BSA. At 30 mM potassium phosphate the recovery yield of DNA was as high as 86% of the total DNA added. The adsorption yield of DNA was about 90%. Therefore, DNA was desorbed almost completely from the particles by the addition of 30 mM potassium phosphate. Similar results were obtained with sodium phosphate and Tris HCl.

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Therefore, DNA sorbed on metal oxide can be desorbed by adding phosphate or Tris ions.

#### EXAMPLE 6

##### 5     Desorption of RNA Sorbed on Metal Oxide Particles

Baker's yeast RNA (0.03% weight/volume) was sorbed to alumina particles (2  $\mu$ m average diameter, 0.17 g particles/mg RNA) prepared according to the method described in EXAMPLE 4 from a mixture with BSA  
10 (0.05%) at 400 mM NaCl in a manner such as that described in EXAMPLE 3. The RNA sorbed on alumina particles was desorbed using potassium phosphate in the manner as described in EXAMPLE 5.

The results are shown in FIG. 8. Plot K  
15 represents RNA and plot L represents BSA. The recovery yield of RNA is higher than 80% of the total RNA added into the system at the concentration of potassium phosphate above 20 mM. The adsorption yield was about 85%. Therefore, RNA can be desorbed and recovered by  
20 adding potassium phosphate.

#### EXAMPLE 7

##### Purification of Plasmid DNA from E. coli with Metal Oxide Particles

Plasmid pUC118 was purified from E. coli  
25 JM83 (described in Gene, 19:259 (1982)) with alumina particles (7  $\mu$ m in diameter) prepared as described in EXAMPLE 4, following the procedure shown in FIG. 1. Ten ml of E. coli JM83 culture in a 15 ml centrifuge tube was spun down and the supernatant was discarded.  
30 Cells were suspended in 1 ml of deionized water and transferred to a microcentrifuge tube. The cells were again spun down and the supernatant was discarded. The cells were resuspended in 0.2 ml of "TGE" buffer (25 mM Tris-HCl, 50 mM glucose, 10 mM EDTA, pH 8.0). The  
35 resuspended cells were lysed by adding 0.4 ml of alkaline lysis reagent (0.2 N NaOH, 1% SDS) and neutralized with 0.4 ml of neutralizing solution (3 M potassium acetate, pH 6.5). Isopropanol (0.6 to 0.7

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ml) was added to the tube to precipitate plasmid DNA from the neutralized cell lysate. The tube was mixed for 1 minute and centrifuged for 1 minute. The supernatant was discarded and the pellet was dissolved in 0.2 ml of deionized water.

A 90% saturated lithium chloride solution in deionized water (0.4 ml, prepared by combining 9 volumes of a saturated solution with 1 volume water) was added and mixed for 15 seconds to precipitate RNA.

10 The tube was spun for 1 minute. The supernatant was transferred to a new microcentrifuge tube and centrifuged again for 1 minute. The supernatant was transferred into a new microcentrifuge tube. The alumina particles (30 to 50  $\mu$ l of 40% suspension in

15 water, pH 2.0 - 4.0) were added into the supernatant of the lithium chloride precipitation, mixed for 15 seconds, and spun for 5 seconds. The supernatant was discarded and the pellet was washed twice with 0.4 ml of deionized water. The plasmid was eluted in 100  $\mu$ l

20 of "TE" buffer (10 mM Tris-HCl, 3 mM EDTA, pH 8.0) by incubating for 5 minutes in a 50°C to 60°C water bath.

The purification of plasmid DNA from the cell pellet was accomplished within 30 minutes without using conventional CsCl gradient ultracentrifugation

25 or phenol extraction. The recovery yield of the plasmid DNA was more than 80%, which was estimated by comparing the intensity of the electrophoresis gel bands. The absorbance ratio (260 nm/280 nm) of the plasmid DNA recovered was higher than 1.93, thereby

30 indicating that the DNA was substantially pure with respect to protein. In order to test residual DNase activity, plasmid DNA recovered was incubated for 18 hours in a 37°C water bath. The incubated plasmid was run on gel and no DNase activity was found.

- 20 -

EXAMPLE 8Purification of Plasmid DNA from  
Cells with Metal Oxide MinicolumnsPlasmid pUC118 was purified from E. coli

5 JM83 with an alumina minicolumn (7  $\mu$ m average particle diameter, prepared according to the method described in EXAMPLE 4) by following the procedure shown in the diagram of FIG. 1. E. coli in 40 ml culture was spun down and resuspended in 4 ml of TGE buffer. The  
10 resuspended cells were lysed by adding 4 ml of alkaline lysis buffer and neutralized with 4 ml of neutralizing solution. The neutralized cell lysate was centrifuged and the supernatant was transferred into a new 50 ml centrifuge tube.

15 One volume of isopropanol was added to the supernatant, mixed until a precipitate formed, and centrifuged. The supernatant was discarded and the pellet was dissolved in 0.4 ml of water. The solution was transferred to a microcentrifuge tube. A 90%  
20 saturated lithium chloride solution (0.8 ml) was added to the centrifuge tube containing the supernatant.

The centrifuge tube was spun for 1 minute and the supernatant was loaded on the alumina minicolumn (0.5 g particles in a 4 ml column). The  
25 column was washed twice with 1 ml of 50% ethanol. The plasmid was eluted with 1 ml of TE buffer or potassium phosphate buffer (50 mM, pH 7.0).

As shown in TABLE 3, as determined spectrophotometrically, the recovery yield of plasmid  
30 was about 70% at the first elution. Additionally, about 20% of plasmid was recovered with the second elution.

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TABLE 3Recovery of Plasmid DNA with a Minicolumn

		<u>Recovery Yield (%)</u>	
	<u>Elution<sup>a</sup></u>	<u>Tris-EDTA</u>	<u>Potassium Phosphate</u>
5	1	69	70
	2	21	20
	3	6	3
	4	3	1
	5	1	0

10

<sup>a</sup> Elution volume: 1 ml per each elution.

EXAMPLE 9

15                      Recovery of DNA from Agarose  
                          Gels with Metal Oxide Particles

Plasmid pUC118 was digested with restriction enzyme Taq I for 2 hours in a 55°C water bath. The digested plasmid (1.2 to 2.3 µg) was run on a 1.5%  
 20 agarose gel in order to separate the various fragment sizes formed. The DNA was recovered from the gel by following the procedure shown in the diagram of FIG. 2. The DNA band was excised using a razor blade and was placed in a microcentrifuge tube. The gel slice  
 25 was melted (i.e., liquefied) by adding 2 to 3 volumes of a gel liquefaction solution, in this case 6 M NaI, although about 4 M to about 8 M NaI would be suitable, and incubating for 2 minutes in a 50°C to 60°C water bath.

30                      To sorb DNA from the gel melt, 1 µl to 10 µl of 40% alumina particle suspension (7 µm average diameter, prepared as according to the method of EXAMPLE 4) was added, mixed for 5 to 8 minutes, and the tube centrifuged for 15 seconds. The pellet was  
 35 washed twice with 0.5 ml of 50% ethanol. DNA was desorbed and eluted in 10 µl of elution buffer (e.g., TE buffer, as described previously) by incubating for 5 to 8 minutes in a 50 to 60° water bath.

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The recovery yield of DNA with alumina particles was compared with those of commercially available glass particles used according to their instructions. "Glass Particle I" was Glassmilk<sup>TM</sup> particles, obtained from Bio101, San Diego, CA, and "Glass Particle II" was US Bioclean<sup>TM</sup> particles obtained from United States Biochemical, Cleveland, OH.

Results are provided in TABLE 4 below, where recovery values were determined as described below. The recovery yield of DNA with alumina particles was as high as 70% of the total DNA added into the gel, while the recovery yield with glass particles was only 50%. When the amount of DNA added was reduced to one-half (0.2  $\mu$ g for 0.5 kb and 0.65  $\mu$ g for 1.4 kb), the recovery yield with alumina particles (higher than 50%) was again much higher than that of the glass particles (30% with glass particle I and 10% with glass particle II). Therefore, the use of alumina particles provides significant advantages, especially when the amount of DNA is limited.

TABLE 4Recovery of DNA from Gel

Size of DNA (kb)	Amount of DNA ( $\mu$ g)	Recovery Yield (%)		
		Alumina	Glass I	Glass II
0.5	0.4	50, 70, 65, 70	50, 50, 50, 50	45, 50, 45, 50
0.5	0.2	50, 55	25, 35	10, 10
0.7	0.6	40, 35, 55	30, 25, 40	30, 20, 40
1.4	1.3	60, 60, 65, 70	50, 45, 60, 60	45, 50, 55, 55
1.4	0.65	55	30	10

In TABLE 4 above, the amount of DNA (micrograms) refers to the estimated amount recovered in the respective gel band. Each number under "Recovery Yield" represents a separate sample, and is



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the average of the value assigned by 3 individuals making visual estimates of intensity of the bands compared to control intensities, in photographic negatives of the gels.

5

EXAMPLE 10

Purification of Plasmid DNA from  
Cells with Alumina-loaded Membranes

Plasmid pUC118 prepared according to the method described in Example 4 above was purified with  
10 an alumina-loaded Empore<sup>TM</sup> membrane. The alumina-loaded Empore<sup>TM</sup> membrane was prepared according to the general method of Example 2 of U.S. Pat. No. 4,906,378. Briefly stated, 25 g of alumina particles (18  $\mu$ m average diameter, prepared according  
15 to the method described in EXAMPLE 4) was combined with 4.6 g of polytetrafluoroethylene resin emulsion (Teflon<sup>TM</sup> 30B available from E. I. DuPont de Nemours & Co., Inc., Wilmington, DE) followed by the addition of about 15 g of 50:50 v/v isopropanol/deionized water.  
20 The resulting putty-like mass was then calendared to provide a membrane with a thickness of approximately 500  $\mu$ m.

E. coli in 10 ml culture was spun down and resuspended in 0.2 ml of TGE buffer. The resuspended  
25 cells were lysed by adding 0.4 ml of alkaline lysis buffer and neutralized with 0.4 ml of neutralizing solution. The neutralized cell lysate was centrifuged and the supernatant was transferred into a new microcentrifuge tube. To the supernatant 0.7 ml of  
30 isopropanol was added, mixed until a precipitate formed, and centrifuged. The supernatant was discarded and the pellet was dissolved in 0.2 ml of water.

A 90% saturated lithium chloride solution (0.4 ml) was added to the centrifuge tube. The  
35 centrifuge tube was mixed and spun for 1 minute. The supernatant was loaded on the spin-filter unit containing 1 to 4 layers of the alumina-loaded membrane. The spin-filter unit was centrifuged for 3

- 24 -

minutes at 6,000 rpm. The membrane was washed twice with 0.4 ml of water by spinning the micro spin-filter unit for 2 minutes at 6,000 rpm. Plasmid DNA was desorbed and eluted with 0.1 ml of TE buffer by  
 5 spinning the micro spin-filter unit for 2 minutes at 6,000 rpm.

As shown in TABLE 5, the amount of plasmid DNA recovered was more than 22  $\mu$ g when two or more layers of membranes were used. The absorbance ratio  
 10 (260 nm/280 nm) of the plasmid DNA recovered was higher than 2.1. This indicated that the plasmid DNA recovered was nearly 100% pure (with respect to protein).

TABLE 5

15                    Recovery of Plasmid DNA with an  
                          Alumina-loaded Membrane

	Number of <u>Membrane Layers</u>	Amount of Plasmid <u>DNA Recovered (<math>\mu</math>g)</u>	Absorbance <u>260/280</u>
	1	4	--
20	2	26	2.11
	3	22	2.18
	4	24	2.13

EXAMPLE 11

25                    Biological Activity of Purified DNA

The biological activity of plasmid DNA purified in the manner described in EXAMPLE 7 was evaluated. Standard enzymatic reactions required for constructing recombinant DNA molecules were evaluated,  
 30 including a variety of restriction endonucleases, calf intestinal phosphatase, polynucleotide kinase and T4 DNA ligase. All of the enzymatic reactions functioned satisfactorily and in each case were equivalent or better than control reactions utilizing DNA purified  
 35 by either a commercially available (CIRCLEPREP<sup>TM</sup>) process or a conventional CsCl gradient centrifugation (as described, for instance, in A Practical Guide to

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Molecular Cloning, B. Perbal, ed., pp. 297-304 (1988)).

The purified plasmid DNA was also able to transform E. coli at a frequency equivalent to 5 plasmids purified by both the commercial process or conventional CsCl gradient centrifugation, achieving a rate of approximately  $10^6$  transformants per microgram plasmid). No detectable damage to the lac Z gene encoded within the plasmid was observed.

10

EXAMPLE 12Biological Activity of DNA Purified from Gels

DNA purified from an agarose gel according to the method described in EXAMPLE 9 was used in the cloning of 1.6 kb Bam H1 generated fragment containing 15 Corynebacterium glutamicum aro A gene. The purified DNA (both the vector and target fragments) was able to form recombinant molecules at an efficiency equivalent or better than that of control DNA purified according to the instructions of a commercial (GENECLEAN<sup>TM</sup>) 20 process.

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What is claimed is:

1. A method for the purification of nucleic acids comprising the steps of

5 (a) releasing substantially all nucleic acids from a biological sample into solution, and

(b) combining said solution with a metal oxide support under conditions in which one or more types of said nucleic acids preferentially sorb to the  
10 support.

2. A method according to claim 1 further comprising the step of desorbing and eluting said sorbed nucleic acid from said metal oxide support.  
15

3. A method according to claim 1 wherein said sample is selected from the group consisting of eucaryotic cells and procaryotic organisms, said sorbed nucleic acid is selected from the group  
20 consisting of linear plasmid DNA, circular plasmid DNA, chromosomal DNA, and RNA, and said metal oxide is selected from the group consisting of the oxides and hydroxides of zirconium and aluminum.

25 4. A method according to claim 3 wherein said metal oxide is alumina.

5. A kit for the purification of nucleic acids comprising

30 a) means for lysing eucaryotic or procaryotic cells in order to release nucleic acids therefrom into solution, and

b) metal oxide support capable of preferentially sorbing said released nucleic acid.  
35

6. A kit according to claim 5 further comprising c) means for desorbing and eluting said sorbed nucleic acid.

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7. A kit according to claim 5 wherein said lysing means comprises i) lysing buffer, ii) alkaline lysis reagent, and iii) neutralizing solution.

5           8. A kit according to claim 5 wherein said support is a metal oxide particle incorporated into a form selected from the group consisting of a column, suspension, coating, and composite structure.

10           9. A kit for the recovery of fragmented and electrophoretically separated nucleic acids from a gel comprising

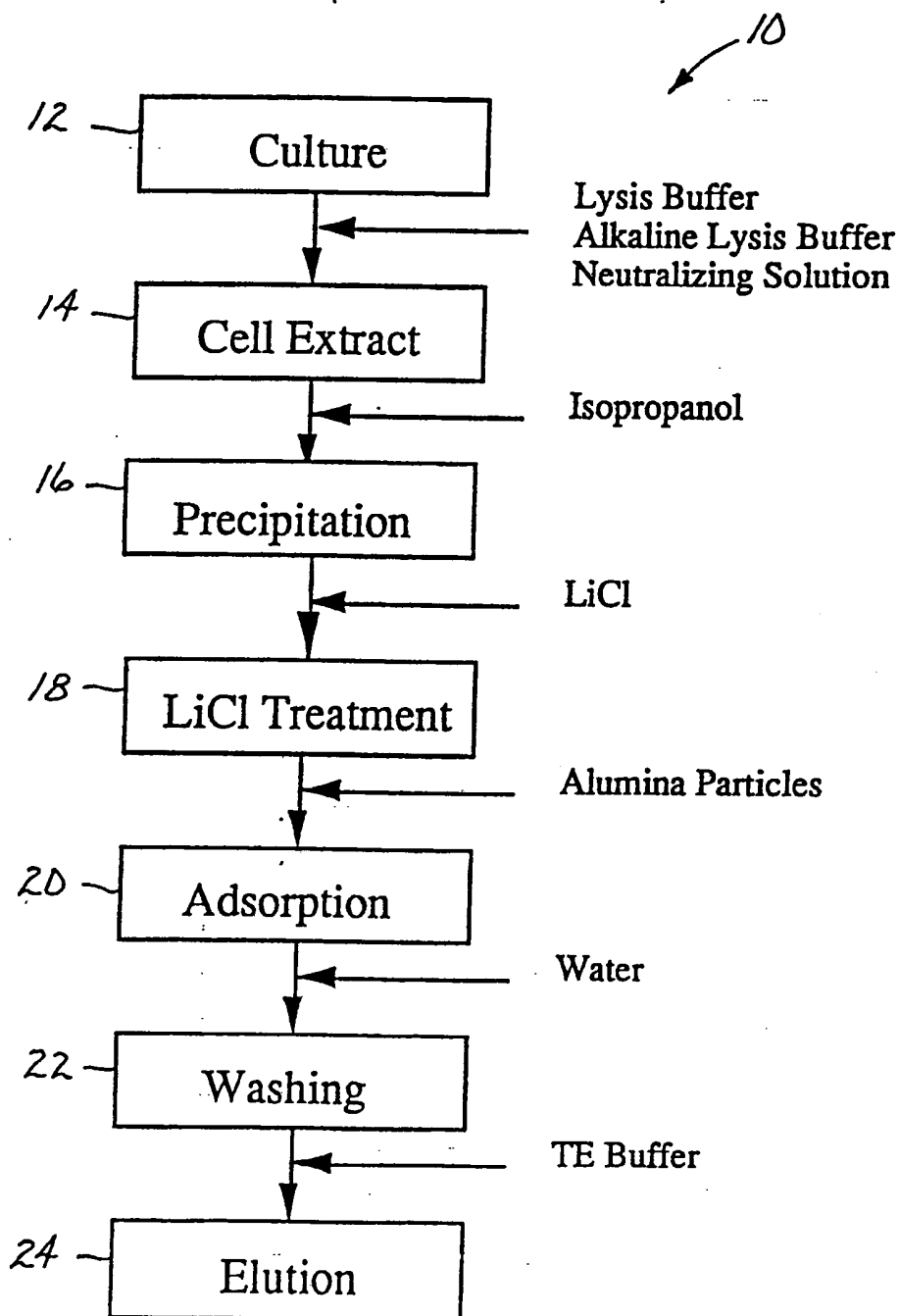
a) means for liquefying said gel,

b) metal oxide support capable of

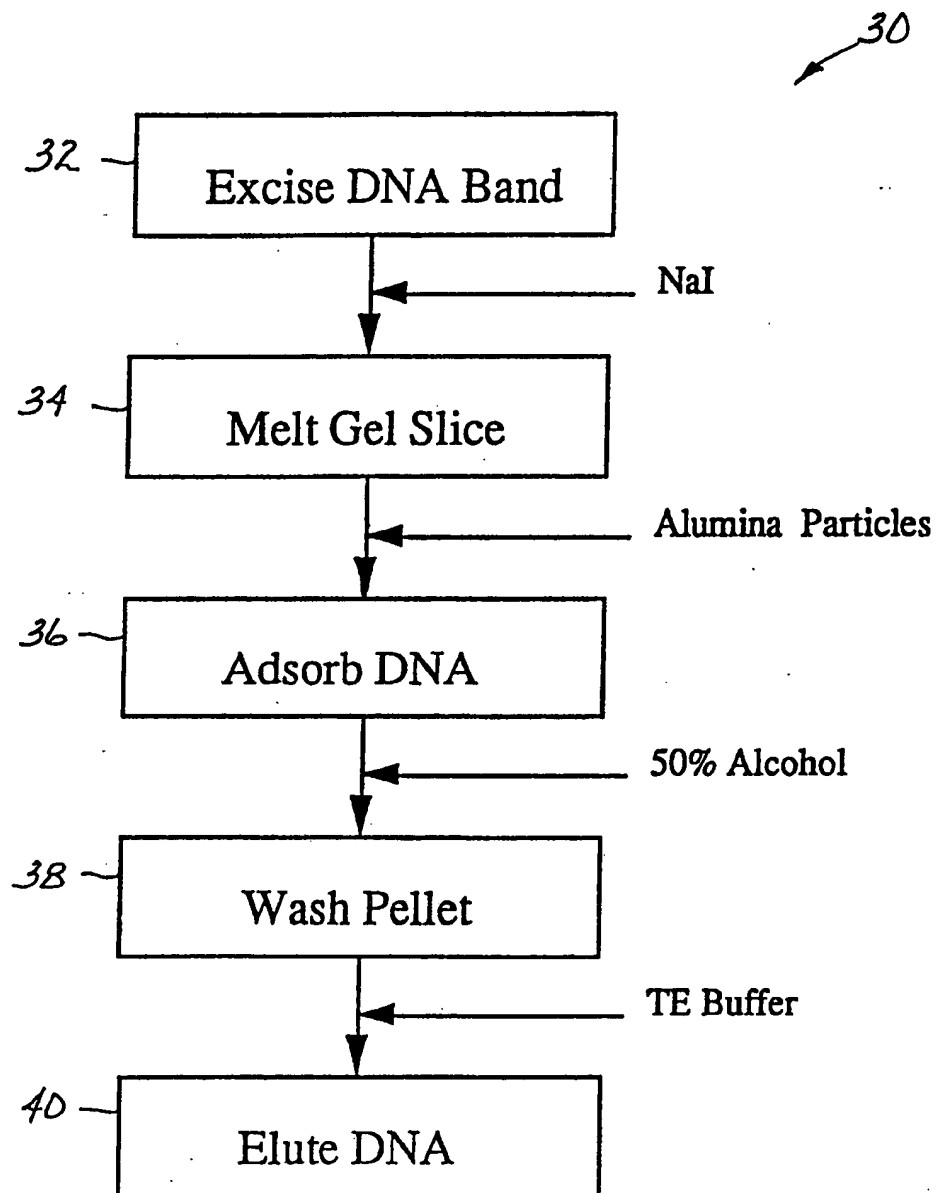
15 preferentially sorbing nucleic acid from gel liquefied by said means, and

c) means for desorbing and eluting nucleic acid sorbed to said support.

20

*Fig. 1*<sup>1/5</sup>**Purification of Plasmid DNA with Particle Suspension**

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*Fig. 2***Recovery of DNA Fragments from Agarose Gel**

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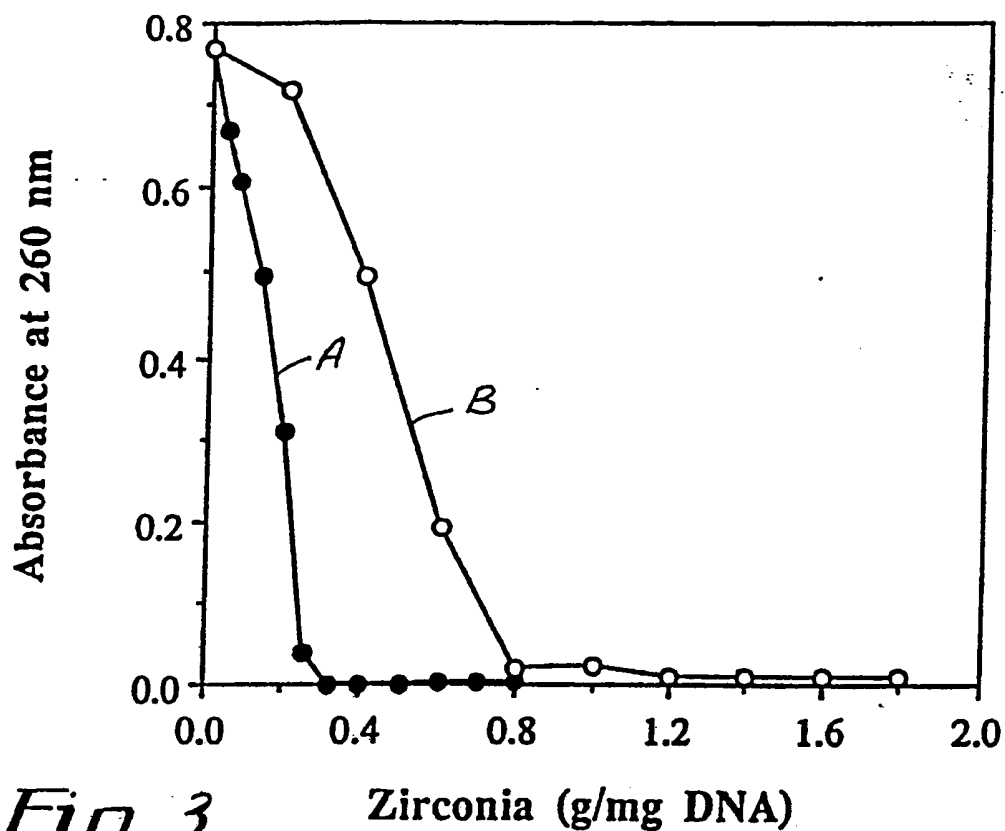


Fig. 3

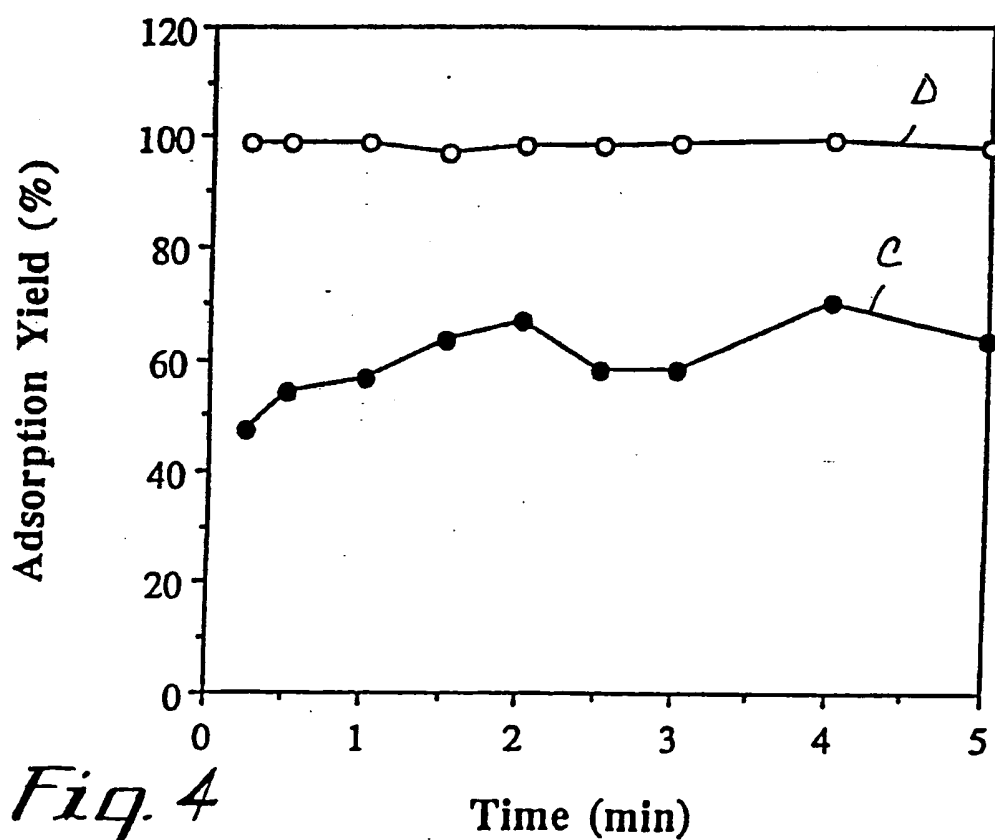
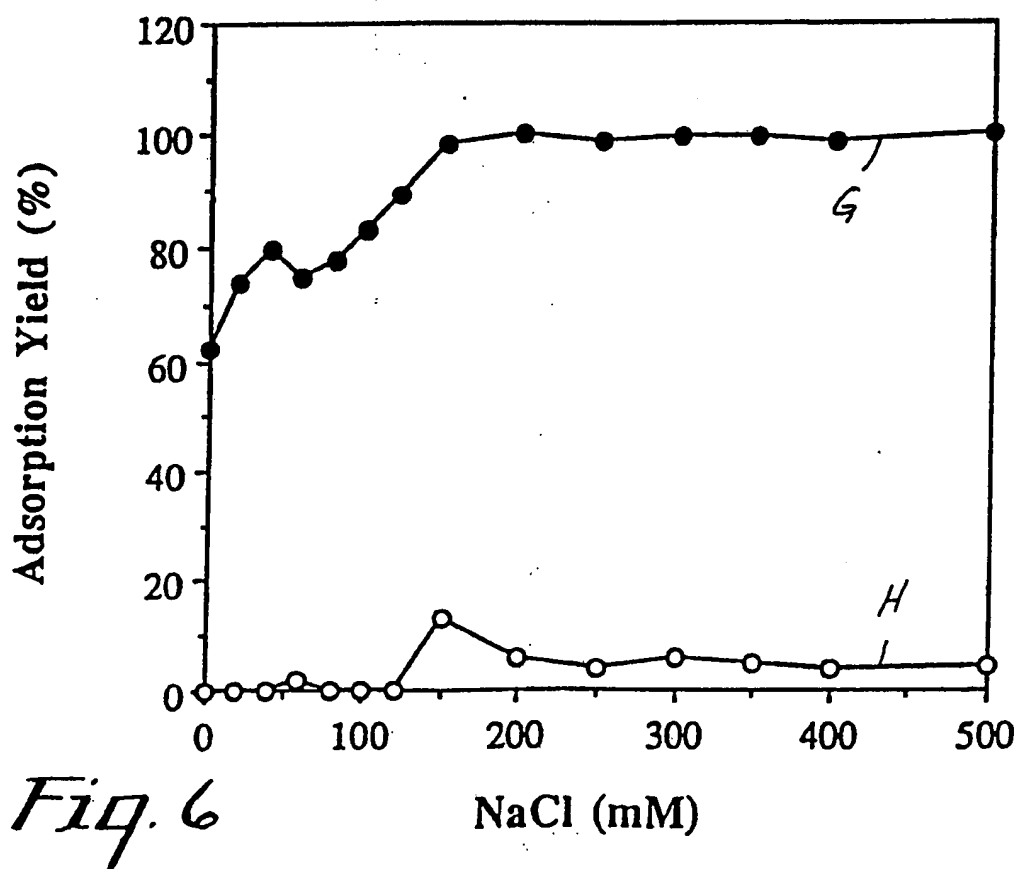
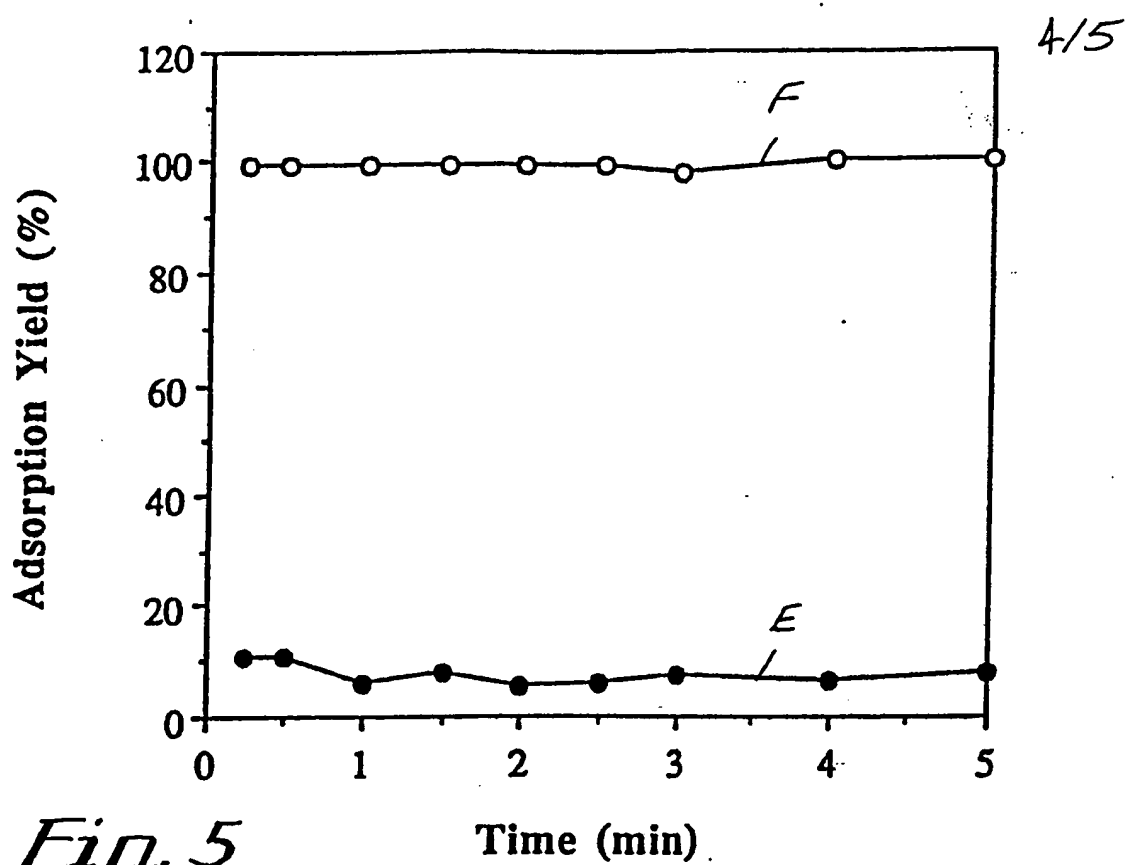
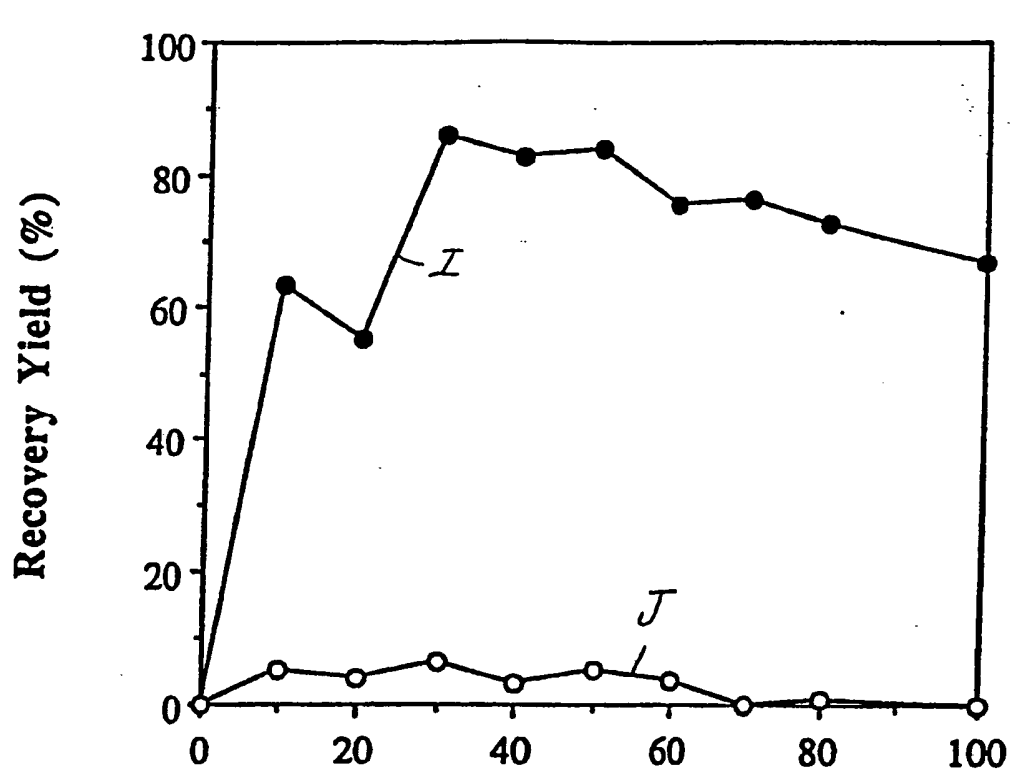


Fig. 4

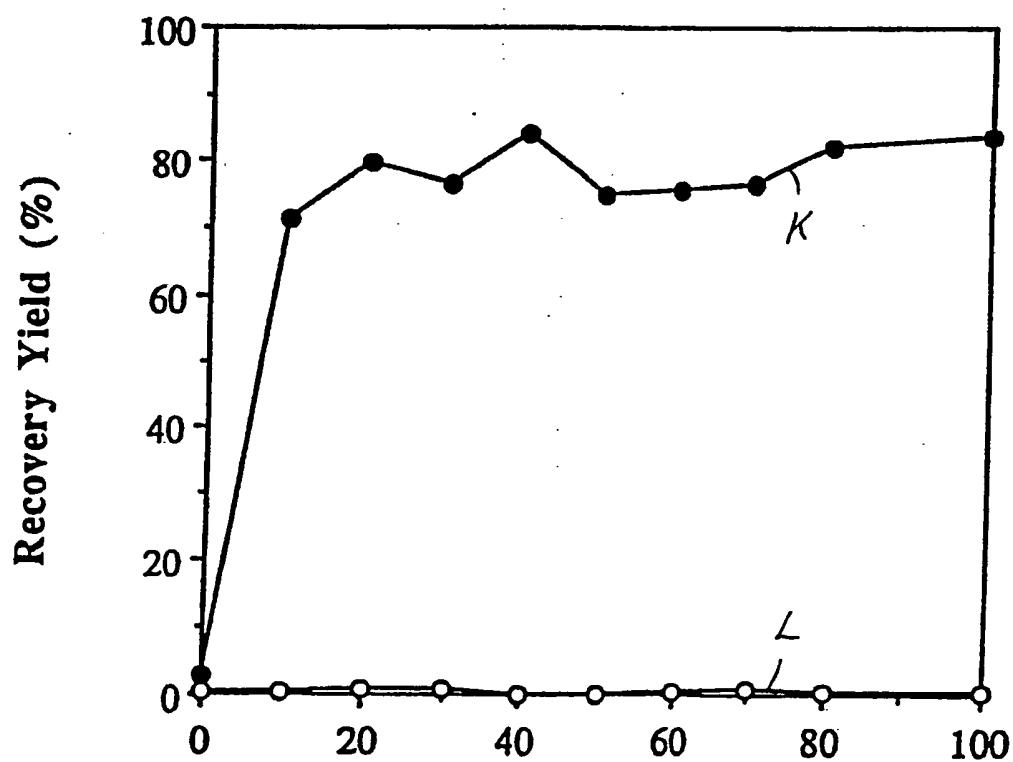
Time (min)







*Fig. 7* Potassium Phosphate (mM)



*Fig. 8* Potassium Phosphate (mM)

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/02262

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>4</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>5</sup> : C 07 H 1/08																				
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched <sup>7</sup></div> <div style="display: flex; justify-content: space-between;"> <div style="width: 30%; border-bottom: 1px solid black; padding-bottom: 5px;">Classification System</div> <div style="width: 70%; border-bottom: 1px solid black; padding-bottom: 5px;">Classification Symbols</div> </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="width: 30%; border-right: 1px solid black; padding-right: 5px;">IPC<sup>5</sup></div> <div style="width: 70%; padding-left: 5px;">C 07 H 1/00, C 07 H 21/00</div> </div> <div style="text-align: center; margin-top: 10px; font-size: small;">         Documentation Searched other than Minimum Documentation          to the extent that such Documents are included in the Fields Searched <sup>8</sup> </div>																				
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <thead> <tr> <th style="width: 10%; text-align: left; padding: 5px;">Category <sup>6</sup></th> <th style="width: 70%; text-align: left; padding: 5px;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; text-align: left; padding: 5px;">Relevant to Claim No. <sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">DD, A3, 230 560 (R.M. LEISER) 04 December 1985 (04.12.85), see the whole document. --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-9</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">EP, A2, 0 268 946 (DIAGEN) 01 June 1988 (01.06.88), see claims. --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-9</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">US, A, 3 652 761 (H.H. WEETALL) 28 March 1972 (28.03.72), see claims; column 3, lines 36-47. --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-4</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">EP, A2, 0 389 063 (AKZO) 26 September 1990 (26.09.90), see claims. --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-9</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">EP, A2, 0 391 608 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-9</td> </tr> </tbody> </table>			Category <sup>6</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	DD, A3, 230 560 (R.M. LEISER) 04 December 1985 (04.12.85), see the whole document. --	1-9	X	EP, A2, 0 268 946 (DIAGEN) 01 June 1988 (01.06.88), see claims. --	1-9	A	US, A, 3 652 761 (H.H. WEETALL) 28 March 1972 (28.03.72), see claims; column 3, lines 36-47. --	1-4	A	EP, A2, 0 389 063 (AKZO) 26 September 1990 (26.09.90), see claims. --	1-9	A	EP, A2, 0 391 608 --	1-9
Category <sup>6</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>																		
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A	EP, A2, 0 391 608 --	1-9																		
<div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p> </div> </div>																				
<b>IV. CERTIFICATION</b> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <td style="width: 50%; padding: 5px;">           Date of the Actual Completion of the International Search   <div style="text-align: center; font-size: large;">24 August 1992</div> </td> <td style="width: 50%; padding: 5px;">           Date of Mailing of this International Search Report   <div style="text-align: center; font-size: large;">15 SEP 1992</div> </td> </tr> <tr> <td style="width: 50%; padding: 5px;">           International Searching Authority   <div style="text-align: center;">EUROPEAN PATENT OFFICE</div> </td> <td style="width: 50%; padding: 5px;">           Signature of Authorized Officer   <div style="text-align: center;">IRMLER e.h.</div> </td> </tr> </table>			Date of the Actual Completion of the International Search  <div style="text-align: center; font-size: large;">24 August 1992</div>	Date of Mailing of this International Search Report  <div style="text-align: center; font-size: large;">15 SEP 1992</div>	International Searching Authority  <div style="text-align: center;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer  <div style="text-align: center;">IRMLER e.h.</div>														
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International Searching Authority  <div style="text-align: center;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer  <div style="text-align: center;">IRMLER e.h.</div>																			

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	(MINNESOTA MINING MANUFACTURE COMPANY) 10 October 1990 (10.10.90), see claims (cited in the application).	
	US, A, 4 648 975 (A. BARKATT et al.) 10 March 1987 (10.03.87), see claims (cited in the application).	1-9

## ANHANG

## ANNEX

## ANNEXE

zum internationalen Recherchen-  
bericht über die internationale  
Patentanmeldung Nr.

to the International Search  
Report to the International Patent  
Application No.

au rapport de recherche inter-  
national relatif à la demande de brevet  
international n°

PCT/US 92/02262 SAE 60627

In diesem Anhang sind die Mitglieder  
der Patentfamilien der im obenge-  
nannten internationalen Recherchenbericht  
angeführten Patentdokumente angegeben.  
Diese Angaben dienen nur zur Unter-  
richtung und erfolgen ohne Gewähr.

This Annex lists the patent family  
members relating to the patent documents  
cited in the above-mentioned inter-  
national search report. The Office is  
in no way liable for these particulars  
which are given merely for the purpose  
of information.

La présente annexe indique les  
membres de la famille de brevets  
relatifs aux documents de brevets cités  
dans le rapport de recherche inter-  
national visée ci-dessus. Les renseigne-  
ments fournis sont donnés à titre indica-  
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Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
DD A3 230560	04-12-85	keine - none - rien	
EP A2 268946	01-06-88	DE A1 3639949 EP A3 268946 JP A2 63150294 US A 5057426	09-06-88 14-03-90 22-06-88 15-10-91
US A 3652761	28-03-72	AT B 309675 BE A1 755775 CA A1 955523 DE A 2042976 DE B2 2042976 FR A5 2060904 GB A 1288328 JP B4 50011448 NL A 7013059	27-08-73 99-99-99 01-10-74 11-03-71 22-11-79 18-06-71 06-09-72 01-05-75 08-03-71
EP A2 389063	26-09-90	AU A1 52153/90 CA AA 2012777 EP A3 389063 JP A2 2289596 NL A 8900725 ZA A 9002190	27-09-90 23-09-90 15-04-92 29-11-90 16-10-90 24-12-91
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